Control of Protein and Sterol Trafficking by Antagonistic Activities of a Type IV P-type ATPase and Oxysterol Binding Protein Homologue

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The oxysterol binding protein homologue Kes1p has been implicated in nonvesicular sterol transport in *Saccharomyces cerevisiae*. Kes1p also represses formation of protein transport vesicles from the *trans*-Golgi network (TGN) through an unknown mechanism. Here, we show that potential phospholipid translocases in the Drs2/Dnf family (type IV P-type ATPases [P4-ATPases]) are downstream targets of Kes1p repression. Disruption of *KES1* suppresses the cold-sensitive (cs) growth defect of *drs2Δ*, which correlates with an enhanced ability of Dnf P4-ATPases to functionally substitute for Drs2p. Loss of Kes1p also suppresses a *drs2-ts* allele in a strain deficient for Dnf P4-ATPases, suggesting that Kes1p antagonizes Drs2p activity in vivo. Indeed, Drs2-dependent phosphatidylserine translocase (flippase) activity is hyperactive in TGN membranes from *kes1Δ* cells and is potently attenuated by addition of recombinant Kes1p. Surprisingly, Drs2p also antagonizes Kes1p activity in vivo. Drs2p deficiency causes a markedly increased rate of cholesterol transport from the plasma membrane to the endoplasmic reticulum (ER) and redistribution of endogenous ergosterol to intracellular membranes, phenotypes that are Kes1p dependent. These data suggest a homeostatic feedback mechanism in which appropriately regulated flippase activity in the Golgi complex helps establish a plasma membrane phospholipid organization that resists sterol extraction by a sterol binding protein.

INTRODUCTION

A fundamental feature of the eukaryotic cell plasma membrane is the asymmetric distribution of phospholipid species between the inner and outer leaflets. Phosphatidylserine (PS) and phosphatidylethanolamine (PE) are highly concentrated in the inner, cytosolic leaflet, with sphingolipids and phosphatidylcholine (PC) enriched in the extracellular leaflet (Balasubramanian and Schroit, 2003). The mechanism for establishing and maintaining phospholipid asymmetry is uncertain; however, type IV P-type ATPases (P4-ATPases) seem to facilitate this process by flipping PS and PE to the cytosolic leaflet (Graham, 2004; van Meer *et al.*, 2008). The budding yeast genome contains five P4-ATPase genes—*DRS2*, *DNF1*, *DNF2*, *DNF3*, and *NEO1*—whereas the human genome contains 14 members of this gene family (Graham, 2004). *NEO1* is an essential gene and the other four members (*DRS2* and the *DNF* genes) form an essential group with partially overlapping functions. Drs2p localizes to the *trans*-Golgi network (TGN) and is required for a flippase activity present in purified TGN membranes with specificity for PS

and PE (Natarajan *et al.*, 2004; Alder-Baerens *et al.*, 2006). Dnf1p and Dnf2p seem to have a substrate preference for PE and PC and are primarily responsible for flippase activity in the yeast plasma membrane (Pomorski *et al.*, 2003; Saito *et al.*, 2004; Elvington *et al.*, 2005). Most of the P4-ATPases interact with a noncatalytic subunit in the Cdc50p family. For example, Drs2p associates with Cdc50p, whereas Dnf1p and Dnf2p associate with Lem3p (also called Ros3p), and these interactions are required for export of the complexes out of the endoplasmic reticulum (ER) and the function of the P4-ATPase (Saito *et al.*, 2004; Chen *et al.*, 2006).

Another well-conserved feature of the eukaryotic plasma membrane is its high concentration of sterol relative to internal organelles. Even though different species produce unique sterols, for example cholesterol in mammals and ergosterol in fungi, the majority of unesterified cellular sterol is localized to the plasma membrane (Daum *et al.*, 1998; Liscum and Munn, 1999). How sterols are concentrated in the plasma membrane is poorly understood. Surprisingly, sterols can move efficiently between the ER, the site of synthesis, and plasma membrane under conditions in which vesicle-mediated protein transport through the secretory pathway is blocked (Kaplan and Simoni, 1985; Li and Prinz, 2004; Baumann *et al.*, 2005). Candidate proteins responsible for this nonvesicular, intracellular sterol transport include the oxysterol binding protein homologues (Osh proteins). These sterol binding proteins include seven members in

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Saccharomyces cerevisiae (Osh1p-Osh7p) and 16 human homologues (Beh *et al.*, 2001). The structure of Osh4p/Kes1p (hereafter referred to as Kes1p) has been solved with sterol present in a deep, hydrophobic binding pocket that is similar to that present in other lipid transfer proteins (Im *et al.*, 2005). Kes1p can transfer sterol between membranes in vitro and inactivation of Kes1p in a strain deficient for the other six Osh proteins perturbs nonvesicular sterol transport in vivo (Im *et al.*, 2005; Raychaudhuri *et al.*, 2006). However, whether Osh proteins mediate bulk sterol transport or function as signaling molecules in response to sterol binding is an important unresolved issue (Fairn and McMaster, 2008).

P4-ATPases and oxysterol binding proteins have also been implicated in vesicle-mediated protein transport (Graham, 2004; Mousley *et al.*, 2007). *DRS2* was recovered in a genetic screen for factors that function with ADP-ribosylation factor (ARF) in vesicle biogenesis from the Golgi (Chen *et al.*, 1999). This screen also recovered *CDC50* (Chen *et al.*, 2006), which encodes a membrane protein that chaperones Drs2p from the ER to the TGN (Saito *et al.*, 2004), the clathrin heavy chain gene (Chen and Graham, 1998), and an auxilin homologue required to uncoat clathrin-coated vesicles (Gall *et al.*, 2000). As implicated from this screen, Drs2p function is required for a subset of ARF and clathrin-dependent pathways mediating protein transport from the TGN to the cell surface, as well as between the TGN and early endosome (Gall *et al.*, 2002; Saito *et al.*, 2004; Furuta *et al.*, 2007; Liu *et al.*, 2008). The Dnf P4-ATPases cannot compensate for the loss of Drs2p in the exocytic and early endosome transport pathways. However, functional overlap between Drs2p and Dnf1p is observed in the transport of proteins from the TGN to late endosomes and the vacuole (Hua *et al.*, 2002).

Kes1p is a negative regulator of vesicle budding in the TGN-early endosomal system, although the mechanistic basis of this repression is not known (Mousley *et al.*, 2007). Loss of function *kes1* alleles were recovered in screens for suppressors of the *kre11* and *sec14* protein trafficking mutants (Jiang *et al.*, 1994; Fang *et al.*, 1996). Kre11p is a component of the TRAPP II nucleotide exchange complex for the Rab proteins Ypt31p and Ypt32p, which regulate vesicle budding from the TGN and early endosomes (Chen *et al.*, 2005; Morozova *et al.*, 2006; Furuta *et al.*, 2007). Sec14p is a yeast phosphatidylinositol/phosphatidylcholine (PI/PC) transfer protein required for exocytic vesicle budding from the TGN (Novick *et al.*, 1980; Bankaitis *et al.*, 1990). Sec14p down-regulates PC synthesis through the CDP-choline pathway (McGee *et al.*, 1994) and also stimulates phosphoinositide synthesis (Rivas *et al.*, 1999), perhaps by presenting substrate to PI 4-kinase (Schaaf *et al.*, 2008). Thus, Sec14p helps generate a membrane composition that is permissive for vesicle budding. Disruption of *KES1* can bypass the essential requirement for Sec14p by a mechanism that is downstream of the CDP-choline pathway for PC synthesis (Fang *et al.*, 1996; Li *et al.*, 2002). Kes1p is recruited to TGN membranes by PI 4-phosphate (PI4P) and may impart an inhibitory influence on vesicle budding by depressing PI4P levels or competing with other effectors for this lipid (Li *et al.*, 2002; Fairn *et al.*, 2007).

Here, we report that *kes1Δ* is also a suppressor of *drs2* alleles, although *kes1Δ* cannot bypass the essential function of the *DRS2/DNF* family of P4-ATPases. Genetically, Kes1p seems to repress Dnf and Drs2p function at the TGN, and we provide biochemical evidence that Kes1p potently antagonizes Drs2p flippase activity in purified TGN membranes. Surprisingly, we also found that Kes1p is hyperactive in *drs2Δ* cells and causes a significant increase in the rate of sterol transport to intracellular membranes. Thus, Drs2p also antagonizes the influence of Kes1p on intracellular ste-

rol transport. These observations imply that a system of checks and balances between a P4-ATPase and oxysterol binding protein controls vesicular and nonvesicular transport processes essential for membrane biogenesis.

MATERIALS AND METHODS

Filipin, nystatin, SDS, calcofluor white (CW), and mevastatin were purchased from Sigma-Aldrich (St. Louis, MO). Papuamide B and Ro09-0198 (Ro-peptide) were generous gifts from Raymond Andersen (University of British Columbia, Vancouver, BC, Canada) and Masato Umeda (Kyoto University, Kyoto, Japan), respectively. [³⁵S]methionine was from PerkinElmer Life and Analytical Sciences (Boston, MA), and the yeast knockout collection was from Invitrogen (Carlsbad, CA).

Media and Strains

Yeast strains used in this study are listed in Supplemental Table 1 and were grown in standard rich medium (YPD) or synthetic defined (SD) minimal media containing the required nutritional supplements (Sherman, 1991). Yeast transformations were performed using a lithium acetate method (Gietz and Woods, 2006). *KES1* was disrupted by polymerase chain reaction (PCR)-mediated replacement with *HIS3* from *Saccharomyces kluyveri* by using primers forward (5'-TCG AAA AAT TTA TAA GAT TTA GTC TCA AGA ATT TCA AGT CCG GAT CCC CGG GTT AAT TAA-3') and reverse (5'-ATT AGT GCA ACG GTA ACA AGT TGT TAC TTT ATC GTT CTC CGA ATT CGA GCT CGT TTA AAC-3') primers with the pFA6a-His3MX6 template as described previously (Longtine *et al.*, 1998). In other strains, *KES1* was replaced with *URA3* by transformation with pRE352 (Fang *et al.*, 1996) digested with EcoRI and BamHI. The BY4741 *kes1Δ drs2Δ* strain was constructed using pZH523 (Hua *et al.*, 2002) to disrupt *DRS2* in the BY4741 *kes1Δ* strain background. For strains used for the cholesterol transport assays, *UPC2* was replaced with *upc2-1* by homologous recombination to allow these strains to take up exogenous cholesterol during aerobic growth (Raychaudhuri *et al.*, 2006).

To test for growth inhibition, cells in early log phase were diluted to 0.1 OD/ml in rich media containing potential inhibitors at various concentrations, and growth was monitored using an enzyme-linked immunosorbent assay plate reader. The OD₆₀₀ after 36 h in rich media without inhibitor was defined as 100% growth for a particular strain and used to normalize data with inhibitor (± SD; n = 6). Tests for growth at high-pressure was done as described previously (Abe and Minegishi, 2008).

Screen for Suppressors of *drs2Δ* and Cloning of *KES1*

Individual colonies of *MATα* and *MATα drs2Δ* strains (SEY6210 *drs2Δ::TRP1* and SEY6211 *drs2Δ::LEU2*) were streaked on rich media and incubated at the nonpermissive growth temperature (20 or 17°C) to select for spontaneous bypass suppressors. Cold-resistant (CR) suppressor strains were backcrossed to parental strains to define recessive and dominant suppressor alleles. Recessive suppressor mutants were intercrossed to define two complementation groups suppressor of *drs2* knockout (*SDK1* 1 and *SDK2*). To clone *SDK1*, strain *BM1001 (drs2Δ sdk1-1)* was transformed with a genomic library (Goodson *et al.*, 1996) and ~50,000 Leu⁺ transformants were replica plated and incubated at 17°C for 5–7 d to screen for cold-sensitive colonies. The genomic library plasmids pBMP1 and pBMP2 were rescued from two cold-sensitive (cs) colonies and were identical in restriction digestion pattern. pBMP1 restored cs growth to *BM1001* upon retransformation and DNA sequencing indicated that it carried an 8.6-kbp fragment of chromosome XVI containing *KES1*.

Microscopy and Immunological Methods

Imaging of cells expressing green fluorescent protein (GFP) fusion proteins and stained with filipin was done as described previously (Beh and Rine, 2004; Liu *et al.*, 2007). Samples for electron microscopy were prepared as described previously (Chen *et al.*, 1999). Sections (50–100 nm) were observed on a CM12 electron microscope (Philips, Eindhoven, The Netherlands). Vesicles (100 nm in diameter) were counted in 50 cell sections for each strain. Error bars are SD (n = 2). Metabolic labeling, immunoprecipitation (Graham, 2001), and immunoblotting (Chen *et al.*, 1999) were performed as described previously. An Odyssey infrared fluorescence detector (LI-COR, Lincoln, NE) was used to quantify Western blots and Coomassie-stained gels.

Phospholipid Translocase Assays

The methods for purifying TGN membranes and assaying Drs2p- and ATP-dependent 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD)-PS translocase activities have been described previously (Natarajan and Graham, 2006). Briefly, equal volumes of purified TGN membranes (0.5 mg/ml), NBD phospholipid (10 μM), and an ATP regenerating system (without ATP) were mixed on ice and then shifted to 37°C to initiate the assay. After 2 h of incubation at 37°C with no ATP, the samples were split and 3 μl of 100 mM ATP or buffer H (10 mM

Figure 1. *kes1* is a suppressor of *drs2Δ* cold-sensitive and high-pressure growth defects. (A) Growth of WT (SEY6210), *drs2Δ* (SEY6210 *drs2Δ*), *drs2Δ sdk1-1* (BMY1001), and *drs2Δ sdk2-64* (BMY1164) strains streaked onto rich medium and incubated at 30 and 17°C. (B) *KES1* confers growth to *drs2Δ sdk1-1*. The WT, *drs2Δ*, and *drs2Δ sdk1-1* strains were transformed with empty plasmid (pRS315), pBMP1 (genomic library clone carrying *KES1*) or a *KES1* subclone (pCTY244) and 10-fold serial dilutions were spotted on minimal medium and incubated at 17°C. (C) Serial dilutions of WT (BY4742), *drs2Δ* (ZHY615M2D), *cki1Δ* (BY4742 YLR133W), *drs2Δ cki1Δ* (KLY891), *sac1Δ* (BY4742 YKL212W), and *drs2Δ sac1Δ* (KLY901) strains were incubated on rich medium at 20°C and 17°C. (D) Equal cell numbers of WT (BY4742), *drs2Δ* (ZHY615M2D), *kes1Δ* (BMY002), and *drs2Δ kes1Δ* (BMY001) strains were seeded in minimal medium and incubated at atmospheric (0.1 mPa) or high pressure (25 MPa) for 10 h at 30°C.

HEPES, pH 7.5, and 150 mM NaCl) was added per 100 μ l of sample. The *kes1Δ* membrane samples were split again and received 1 μ l of Kes1p or buffer H per 100 μ l of sample. The samples were incubated an additional 2 h at 37°C. Aliquots of membranes were removed at 0, 2 (before ATP addition), and 4 h of incubation and NBD-PS in the cytosolic leaflet was extracted onto fatty-acid free BSA and quantified. The difference in the percentage of NBD-PS in the cytosolic leaflet of wild-type (WT) TGN membranes incubated with or without ATP at the 4-h time point was defined as 100% NBD-PS flippase activity and was used to normalize data for other conditions assayed. Assays were performed in duplicate and averages from at least three independent experiments ($\pm$ SD) are reported. Quantification of ergosterol in *kes1Δ* TGN membranes was done as described previously (Hanover *et al.*, 2005).

RESULTS

kes1 Suppresses *drs2Δ* Cold-sensitive Growth

Cells harboring knockout alleles of *DRS2* exhibit a striking cold-sensitive growth defect. Wild-type yeast can grow over a range of temperatures from 10 to 40°C, whereas *drs2Δ* strains can only grow over a temperature range of 21–40°C. To explore the mechanism underlying this extreme cs growth defect, we carried out a selection for spontaneous extragenic suppressors that allowed growth of *drs2Δ* at 17°C. Complementation tests indicated that the recessive *drs2Δ* suppressor mutants fell into two complementation groups, which we named *SDK1* and *SDK2*. The *SDK1* and *SDK2* groups are composed of 12 and four recessive extragenic bypass suppressors of *drs2Δ*, respectively. Figure 1A shows the 30 and 17°C growth phenotypes of representative *drs2Δ sdk1* and *drs2Δ sdk2* mutants relative to parental *drs2Δ* and WT strains. Surprisingly, the *drs2Δ sdk1* double mutant formed larger colonies than even the WT strain at 17°C (Figure 1B).

SDK1 Was Cloned by Complementation of the Cold-resistant Growth Phenotype of *drs2Δ sdk1-1*

A genomic library plasmid (pBMP1) was isolated that was able to complement *sdk1-1* upon retransformation of *drs2Δ sdk1-1* cells (Figure 1B). Among the five open reading frames in pBMP1, *KES1* was the most likely candidate for the *sdk1*-complementing gene and as expected, a *KES1* subclone complemented the cold-resistant phenotype of *drs2Δ sdk1-1* (Figure 1B). Several of the *kes1* alleles isolated in the *sec14* suppressor screen contained nonsense mutations, leading to the expression of truncated, nonfunctional proteins (Fang *et al.*, 1996; Li *et al.*, 2002). We examined the expression of Kes1p in the *drs2Δ sdk* mutants by Western blotting and found that seven of the 12 *sdk1* alleles failed to express full-length Kes1p, suggesting that they also carry nonsense mutations (Supplemental Figure 1). These data indicate that *sdk1* strains carry mutations in *KES1* (i.e., *SDK1* and *KES1* are the same gene) and that loss of function *kes1* mutations suppresses *drs2Δ* cs growth. Kes1p is one of seven oxysterol binding protein homologues in yeast. None of the other six members were able to restore a cs growth phenotype to either *drs2Δ sdk1-1* or *drs2Δ sdk2*, even when overexpressed from multicopy plasmids (data not shown). We have not yet succeeded in cloning *SDK2*; so, this report focuses on *SDK1/KES1*.

Mutations in three genes (*CKI1*, *PCT1*, and *CPT1*) encoding enzymes of the CDP-choline pathway involved in phosphatidylcholine biosynthesis can bypass the essential requirement of *SEC14*. However, we found that disruption of *CKI1* in the *drs2Δ* background failed to suppress cs growth of *drs2Δ* (Figure 1C). Hence, inactivation of phosphatidylcholine biosynthesis via the CDP-choline pathway does not

exert bypass suppression of *drs2Δ*. Deletion of *SAC1*, a phosphoinositide phosphatase, increases PI4P levels and suppresses *sec14-ts* (Rivas *et al.*, 1999). *SAC1* deletion suppressed the cold sensitivity of *drs2Δ* at 20°C but not at 17°C (Figure 1C). However, the *sac1Δ* single mutant also failed to grow at 17°C and so the lack of *drs2Δ* suppression at this temperature may not be significant.

A high percentage of *Saccharomyces* mutants that cannot grow at low temperature, including *drs2Δ*, also fail to grow at high hydrostatic pressure (Abe and Minegishi, 2008). This strong positive correlation suggests that the mutants have a defect in membrane order and fluidity. To determine whether *kes1Δ* would also suppress the high-pressure growth defect of *drs2Δ* at 30°C, we measured the growth of WT, *drs2Δ*, *kes1Δ*, and *drs2Δ kes1Δ* strains at atmospheric pressure (0.1 MPa) and high pressure (25 MPa; ~250 kg/cm²) in liquid medium over a 10-h period. Under these conditions, *drs2Δ* grew slower than WT or *kes1Δ* cells at 0.1 MPa, and this growth defect was exacerbated at 25 MPa. At both pressure conditions, the *drs2Δ kes1Δ* cells showed improved growth, indicating that the *drs2Δ* high-pressure sensitivity was at least partially suppressed by *kes1Δ* (Figure 1D).

kes1Δ Suppression of *drs2Δ* Requires DNF P4-ATPase Genes

Drs2p is part of an essential group of P4-ATPases that also includes Dnf1p, Dnf2p, and Dnf3p (Hua *et al.*, 2002). In the absence of Dnf P4-ATPases (*dnf1,2,3Δ* cells), Drs2p can support growth of yeast over the full temperature range by itself, but the *drs2Δ dnf1,2,3Δ* quadruple mutant is dead at any temperature (Hua *et al.*, 2002). To determine whether *kes1Δ* could bypass the essential requirement for *DRS2/DNF* function, or whether the mechanism of suppression required the presence of Dnf ATPases, we tested whether deletion of *KES1* would rescue viability of a *drs2Δ dnf1,2,3Δ* strain. A strain was generated that carries null alleles of all four members of the *DRS2/DNF* group by first introducing a wild-type copy of *DRS2* on a *URA3*-based plasmid (*pURA3-DRS2*) (Hua *et al.*, 2002). This quadruple mutant strain (*drs2Δ dnf1,2,3Δ*) could not lose *pURA3-DRS2* and grow on 5-fluoro-orotic acid (5-FOA) (Figure 2A), a compound that kills cells retaining *URA3*-based plasmids. In contrast, WT and *drs2Δ* cells were able to lose the *pURA3-DRS2* plasmid and form colonies on 5-FOA plates. Importantly, the *drs2Δ dnf1,2,3Δ kes1Δ* strain failed to grow on the 5-FOA plate (Figure 2A). Therefore, *kes1Δ* suppression of *drs2Δ* requires one or more Dnf P4-ATPase.

To determine which Dnf proteins are required to support suppression, we tested the ability of *kes1Δ* to suppress different combinations of *drs2Δ* and *dnfΔ* alleles (Figure 2B). Growth defects of a *drs2Δ dnf1Δ* strain at 30 and 17°C were efficiently suppressed by *kes1Δ*, indicating that *DNF2*, *DNF3*, or both can support *kes1Δ* suppression. A strain expressing only *DNF2* was weakly suppressed, whereas a strain carrying only *DNF1* exhibited a WT level of suppression. We were unable to recover a *drs2Δ dnf1,2Δ kes1Δ* strain expressing only *DNF3*. However, the strain with wild-type *DNF2* and *DNF3* was suppressed better than the strain carrying only *DNF2*, indicating some influence from *DNF3*. Therefore, we conclude that all three Dnf ATPases contribute to the suppression of *drs2Δ* by *kes1Δ*. We also tested whether *kes1Δ* could suppress a temperature-sensitive (*ts*) for function allele of *drs2* in the absence of the Dnf ATPases. Disruption of *KES1* restored a wild-type growth rate at 37°C to a *drs2-ts dnf1,2,3Δ* strain (Figure 2C), and so loss of Kes1p can improve the function of a crippled Drs2p.

Figure 2. Specificity for *kes1Δ* suppression of P4-ATPase mutants. (A) *kes1Δ* cannot bypass the essential function of the *DRS2-DNF* gene family. Strains harboring the indicated combination of null (Δ) and WT (+) alleles for P4-ATPase and *KES1* genes, and carrying wild-type *DRS2* on a *URA3* plasmid, were spotted on minimal media with and without 5-FOA. Strains used were BY4742, ZHY615M2D, ZHY704, and BMY04. (B) Strains harboring the indicated combination of null (Δ) and WT (+) alleles were spotted on rich medium and incubated at 30 and 17°C. Strains used were BY4742, ZHY615M2D, ZHY2149D, BMY05, ZHY708, BMY029, ZHY7282C, and BMY039a. (C) *kes1Δ* suppresses the temperature-sensitive growth defect of a *drs2-ts dnf1,2,3Δ* strain at 37°C. Strains used were ZHY409, ZHY410-3A, and BMY041.

Kes1p Attenuates Drs2p Flippase Activity at the TGN

The ability of *kes1Δ* to suppress *drs2-ts* in the *dnf1,2,3Δ* background suggests that Kes1p antagonizes Drs2p activity at the Golgi complex. To test this possibility biochemically, TGN membranes were purified from WT, *drs2Δ*, *kes1Δ*, and *drs2Δ kes1Δ* cells to assay for phospholipid translocase (flippase) activity. Equal amounts of protein from each TGN preparation were first probed for Kes1p by immunoblot to determine whether Kes1p copurifies with these membranes (data for WT membranes is shown in Figure 3A). Relative to the recombinant Kes1p standard, the TGN membranes from WT and *drs2Δ* cells carry ~2 ng (40 fmol) of Kes1p per 3 μg of total protein. From similar quantitative immunoblots, the WT and *kes1Δ* membranes contain ~0.6 ng of Drs2p (4 fmol) per 3 μg of total protein.

Each TGN membrane preparation was assayed for NBD-PS translocase activity as described under *Materials and Methods*. The ATP-dependent translocation (flip) of NBD-PS from the luminal leaflet to the cytosolic leaflet was quantified and normalized to the WT membrane sample. The *kes1Δ* membranes displayed significantly greater NBD-PS flippase activity than WT membranes. This flippase activity was eliminated in the *drs2Δ* membrane sample and was substantially reduced in *drs2Δ kes1Δ* membranes (Figure 3B).

Figure 3. Kes1p represses the flippase activity of Drs2p in TGN membranes. (A) TGN membranes were purified from WT (BY4742), *kes1Δ* (BMYO2), *drs2Δ* (ZHY615M2D), and *drs2Δ kes1Δ* (BMYO1) cells. The indicated amounts of WT TGN membranes and recombinant Kes1p were immunoblotted to quantify Kes1p in this membrane sample. (B) ATP-dependent translocation of NBD-PS from the luminal leaflet to the cytosolic leaflet of the TGN membranes (flippase activity) was measured as described under *Materials and Methods*. NBD-PS flippase activity in WT membranes was defined as 100% activity and used to normalize data for other samples. Error bars are SD (n = 3; **p = 0.005; *p ≤ 0.0223 compared with *kes1Δ* membranes using Student's *t* test). (C) Kinetics of NBD-PS translocation by Drs2p. Golgi membranes from WT (closed symbols) and *kes1Δ* (open symbols) cells were incubated without ATP for 4 h to allow passive translocation of NBD-PS into the inner leaflet. ATP was then added and the amount of NBD-PS flipped to the outer (cytosolic) leaflet in 15 min was measured as described under *Materials and Methods*.

Thus, Drs2p was primarily responsible for the enhanced NBD-PS flippase activity in *kes1Δ* membranes. The Dnf P4-ATPases may have contributed the small increase in *drs2Δ kes1Δ* NBD-PS flippase activity relative to *drs2Δ* membranes, although these data are not statistically different.

It was formally possible that the enhanced NBD-PS flippase activity in *kes1Δ* membranes was an indirect effect of the chronic Kes1p deficiency. Therefore, we tested whether adding back recombinant Kes1p to the mutant membranes would repress the Drs2-dependent flippase activity. Addition of recombinant Kes1p to the *kes1Δ* TGN membranes attenuated NBD-PS flippase activity to wild-type levels (Figure 3B). Remarkably, the half-maximal inhibitory concentration was in the range of 1 to 10 pg of Kes1p per 17 μg of total Golgi protein (0.02–0.2 pM Kes1p). For comparison, the WT membrane preparation contained ~10 ng of endogenous Kes1p and addition of up to 1 μg of recombinant Kes1p to the *kes1Δ* membranes conferred no additional inhibition of NBD-PS flippase activity (unpublished observation). We considered the possibility that the recombinant Kes1p was

attenuating Drs2p activity by extracting ergosterol from the TGN membrane. However, the *kes1Δ* TGN membranes used for these assays contained 60 ± 15 μg of ergosterol per milligram of protein. At 0.1 ng of Kes1p, the Kes1p to ergosterol stoichiometry would be 1:10,000; so, it is unlikely that this concentration of Kes1p would significantly alter the ergosterol content of the TGN membrane.

The NBD-PS flippase activity shown in Figure 3B was determined after 2 h of incubation with ATP, at which time all the NBD-PS probe in the *kes1Δ* samples was flipped to the cytosolic leaflet, and so the flippase assay was saturated. To better assess the influence of Kes1p on the kinetics of NBD-PS translocation, we measured the amount of NBD-PS flipped in 15 min of incubation with ATP (Figure 3C). After initial incorporation of the NBD-PS probe in the cytosolic leaflet, WT and *kes1Δ* TGN membranes were incubated for 4 h without ATP to allow spontaneous redistribution of ~40% of the probe to the luminal leaflet (NBD-PS that is resistant to back-extraction with fatty-acid free BSA). ATP was then added and the membranes were incubated at 37°C for 15 min before back-extraction of the probe with BSA. Again, nearly all of the NBD-PS was flipped to the cytosolic leaflet of the *kes1Δ* membranes in 15 min. Under these conditions, we estimate that ~345 NBD-PS molecules were flipped per Drs2p molecule per minute in the *kes1Δ* membranes, relative to ~160 NBD-PS molecules flipped/Drs2p/min in WT membranes.

Drs2p Antagonizes the Influence of Kes1p on Sterol Trafficking and Subcellular Distribution

Because Kes1p binds ergosterol and is implicated in the intracellular transport of this sterol, we examined the relationship of *drs2Δ* suppression by *kes1Δ* to ergosterol metabolism and localization. We found that *drs2Δ* cells are hypersensitive to mevastatin (Figure 4A) an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme of ergosterol synthesis. *kes1Δ* cells are slightly hypersensitive to mevastatin, but the *drs2Δ kes1Δ* strain exhibits WT resistance. Perturbation of TGN function in *drs2Δ* cells also alters the trafficking, function, or both of cell wall biosynthetic enzymes. This causes hypersensitivity of *drs2Δ* to CW, a chitin-binding compound that can interfere with cell wall assembly. By contrast to the mevastatin hypersensitivity, *kes1Δ* does not suppress the CW hypersensitivity of *drs2Δ* (Figure 4A). *kes1Δ* also completely suppressed the hypersensitivity of *drs2Δ* to nystatin (Figure 4B), a polyene antifungal compound that binds ergosterol in the plasma membrane.

drs2Δ is synthetically lethal with *erg6Δ*, a gene encoding the Δ(24)-sterol C-methyltransferase that converts zymosterol to fucosterol in the ergosterol biosynthetic pathway (Kishimoto *et al.*, 2005). Viability of a *drs2Δ erg6Δ* strain can be sustained with WT *DRS2* carried on a *URA3* plasmid (*pURA3-DRS2*). This strain cannot lose *pURA3-DRS2* and therefore failed to form colonies on media containing 5-FOA, whereas the other single and double mutants grew well (Figure 4C). However, a *drs2Δ erg6Δ kes1Δ* strain readily lost the *pURA3-DRS2* plasmid and grew robustly on 5-FOA. Thus, *kes1Δ* suppresses the synthetic lethality between *drs2Δ* and *erg6Δ*. The data shown in Figure 4, A–C, suggests that *drs2Δ* cells might have a defect in ergosterol synthesis. However, a normal concentration of ergosterol in *drs2Δ* cells has been reported previously (Fei *et al.*, 2008), and we have confirmed this result (data not shown).

To determine whether *drs2Δ* perturbs ergosterol subcellular distribution, we stained cells with filipin, a fluorescent polyene antifungal compound that also binds ergosterol. Filipin primarily stained the plasma membrane of WT cells

(Figure 4D), where most cellular ergosterol is localized. In contrast, substantial intracellular filipin staining was observed with *drs2Δ* cells (Figure 4D). *kes1Δ* cells also exhibited more intracellular filipin staining than WT cells. However, filipin primarily stained the plasma membrane of *drs2Δ kes1Δ* cells (Figure 4D). In this case, we observed cosuppression because the *drs2Δ kes1Δ* double mutant seemed more similar to WT than either single mutant.

Previous studies indicated that inactivation of Kes1p/Osh4p perturbs transport of sterols from the plasma membrane to the ER (Raychaudhuri *et al.*, 2006). The toxicity of Kes1p to *drs2Δ* and the enhanced intracellular filipin staining in this mutant suggested the possibility that Kes1p is hyperactive in transporting sterol from the *drs2Δ* plasma membrane to internal organelles. To test this hypothesis, we compared the rate of esterification for exogenously supplied radiolabeled cholesterol in WT, *drs2Δ*, *kes1Δ*, and *drs2Δ*

kes1Δ cells. Esterification of cholesterol requires its transport from the plasma membrane, where it is taken up, to the ER, where the acyl-CoA:cholesterol acyltransferases (ACATs) are localized. *S. cerevisiae* will not normally take up exogenous sterol in aerobic conditions. Therefore, we introduced an altered allele of a transcription factor (*upc2-1*) into our strains to permit aerobic sterol uptake (Raychaudhuri *et al.*, 2006). After incubation with [¹⁴C]cholesterol, the cells were harvested at the times indicated in Figure 5, and the amount of free cholesterol and cholesteryl ester were measured. The *drs2Δ* cells transported cholesterol from the plasma membrane to the ER 5 to 6 times faster than WT cells at 30°C, and three times faster than WT at 20°C (Figure 5, cholesteryl ester). The *drs2Δ* cells also took up significantly more cholesterol (free) than WT cells. In contrast, the *drs2Δ kes1Δ* cells took up cholesterol and transported it to the ER at rates indistinguishable from the WT cells. Therefore, Kes1p was

Figure 6. *kes1Δ* does not suppress *drs2Δ* loss of plasma membrane phospholipid asymmetry. WT, *drs2Δ*, *kes1Δ*, and *drs2Δ kes1Δ* strains (same as in Figure 3C) were subcultured to 0.1 OD₆₀₀/ml in rich media with or without papuamide B, Ro090198 (Ro peptide), or SDS and incubated at 30°C for 36 h. Percentage of growth was determined as described in Figure 4B.

required for the markedly enhanced rate of cholesterol transport in *drs2Δ* cells.

Phospholipid asymmetry of the plasma membrane is also perturbed in *drs2Δ* cells (Pomorski *et al.*, 2003; Chen *et al.*, 2006) and we tested whether *kes1Δ* would suppress this defect. PS and PE, which are normally restricted to the inner, cytosolic leaflet, are aberrantly exposed on the outer leaflet of *drs2Δ* cells. This loss of asymmetry makes *drs2Δ* hypersensitive to papuamide B and Ro, antifungal compounds that permeabilize cells exposing PS or PE, respectively. Disruption of membrane integrity can also make cells hypersensitive to membrane permeating agents; so, sensitivity to low concentrations of SDS was also examined to control for general effects of the mutations on membrane integrity. *kes1Δ* partially suppressed the SDS, papuamide B, and Ro sensitivity of *drs2Δ*, but the double mutant remained significantly more sensitive to papuamide B and Ro than WT cells (Figure 6). We conclude that loss of Kes1p improves the plasma membrane integrity of *drs2Δ* cells, presumably by restoring sterol content, but it does not restore the normal asymmetric distribution of PS and PE. Surprisingly, *kes1Δ* was also hypersensitive to papuamide B and Ro, yet resistant to SDS, indicating a partial loss of membrane asymmetry. This observation suggests that hyperactivity of P4-ATPases may also disrupt normal plasma membrane organization.

kes1Δ Does Not Suppress *drs2Δ* Defects in Protein Trafficking between the TGN, Plasma Membrane, and Early Endosomes

The suppression of *sec14-ts* secretion defects by *kes1Δ* suggested that the protein trafficking defects of *drs2Δ* would also be suppressed by *kes1Δ*. Therefore, we examined the ability of *kes1Δ* to suppress trafficking defects in several distinct pathways caused by *drs2Δ*. Loss of Drs2p function perturbs formation of one class of exocytic vesicles that rely on actin cables for efficient transport to the bud plasma membrane (Gall *et al.*, 2002). The *sla2Δ* mutation interferes with actin assembly and causes an accumulation of an average of 10 post-Golgi transport vesicles per cell section in electron micrographs. However, *sla2Δ drs2Δ* cells have only an average of one to two vesicles per cell section, comparable with WT cells, indicating that *drs2Δ* is epistatic to *sla2Δ*

for the vesicle accumulation phenotype and is essential for budding these vesicles at 30°C (Figure 7A; Gall *et al.*, 2002). Surprisingly, *kes1Δ* did not suppress the *drs2Δ* defect in budding in these post-Golgi exocytic vesicles because the *sla2Δ drs2Δ kes1Δ* cells contained about the same low number of vesicles as *sla2Δ drs2Δ* cells (Figure 7A).

Loss of Drs2p function also perturbs trafficking of Snc1p, an exocytic SNARE, which normally cycles in a TGN → plasma membrane → early endosome → TGN loop. In wild-type cells, Snc1-GFP primarily localized to the bud plasma membrane, whereas in *drs2Δ* Snc1-GFP was mislocalized to internal punctate structures (Figure 7B; Hua *et al.*, 2002), reflecting a defect in the early endosome to TGN transport step (Saito *et al.*, 2004). We found no difference in the localization of Snc1-GFP to intracellular punctate structures in *drs2Δ* and *drs2Δ kes1Δ* cells (Figure 7B). We also found no evidence for *kes1Δ* suppression of the *drs2Δ* defect in AP-1-dependent trafficking of chitin synthase between the TGN and early endosome (Supplemental Figure 2).

kes1Δ Suppresses *drs2Δ* Defects in TGN to Vacuole Protein Transport

The protein trafficking steps examined above are disrupted at high temperatures (30–37°C) as well as low temperatures in *drs2Δ* cells. In contrast, transport of carboxypeptidase Y (CPY) from the Golgi to the vacuole is kinetically delayed in *drs2Δ* cells at low temperatures but is normal at 30°C (Chen *et al.*, 1999). We tested whether *kes1Δ* would suppress this cold-sensitive defect in CPY transport kinetics by using pulse-chase analyses. CPY is initially synthesized in the ER as a 67-kDa p1 precursor form that is further modified in the Golgi to the 69 kDa p2 precursor. p2 CPY is then sorted from secretory cargo at the TGN, transported to the late endosome and on to the vacuole where it is processed to the 61-kDa mature form (mCPY) (Stevens *et al.*, 1982). WT, *drs2Δ*, *kes1Δ*, and *drs2Δ kes1Δ* strains were labeled with ³⁵S-amino acids and chased at 30 and 15°C for the times indicated in Figure 8A. At 30°C, no significant difference was found for CPY maturation kinetics in these four strains. In contrast, *drs2Δ* cells exhibited a threefold kinetic delay in CPY transport at 15°C relative to WT and *kes1Δ* cells (Figure 8A). This cold-sensitive CPY transport defect was completely suppressed in the *drs2Δ kes1Δ* cells.

Figure 7. *kes1Δ* does not suppress *drs2Δ* defects in protein trafficking between the TGN, plasma membrane, and early endosome. (A) *kes1Δ* does not suppress the *drs2Δ* defect in budding post Golgi exocytic vesicles. WT (SEY6210), *sla2Δ* (TGY1912), *drs2Δ sla2Δ* (CCY642), and *drs2Δ sla2Δ kes1Δ* (BMY15) strains were grown to mid-log phase in rich media at 30°C and processed for electron microscopy as described previously. Eighty- to 100-nm vesicles were counted in 50 sections for each strain. Error bars are SD (n = 2). (B) *kes1Δ* does not suppress the Snc1p recycling defect of *drs2Δ* cells. WT, *drs2Δ*, *kes1Δ*, and *drs2Δ kes1Δ* cells (same strains as Figure 4C) expressing GFP-Snc1p were grown to mid-log phase at 30°C and viewed by fluorescence microscopy.

In addition, p2 CPY was not fully formed in *drs2Δ* cells at 15°C (p1 and p2 CPY were not completely resolved by SDS-PAGE), indicating a partial defect in a terminal mannosylation event catalyzed within late Golgi compartments (Chen *et al.*, 1999). This cold-sensitive glycosylation defect was also completely suppressed by *kes1Δ*. We also examined the transport kinetics of carboxypeptidase S (CPS), another vacuolar protein that seems to follow the same route to the vacuole as CPY (Costaguta *et al.*, 2001). The *drs2Δ* cells also showed a cs, 3-fold kinetic defect in the transport of CPS to the vacuole that was suppressed by *kes1Δ* (data not shown).

Drs2p and Dnf P4-ATPases have a redundant function in the transport of CPY and alkaline phosphatase (ALP) to the vacuole (Hua *et al.*, 2002). In contrast to CPY and CPS, ALP seems to be transported directly from the Golgi to the vacuole without passing through an endosomal intermediate. ALP vacuolar transport is weakly perturbed by *drs2Δ*, but a strong defect is observed in *drs2Δ dnf1Δ* double mutants. To test for *kes1Δ* suppression of this transport defect, we examined the localization of ALP-GFP in WT, *drs2Δ dnf1Δ*, and *drs2Δ dnf1Δ kes1Δ* cells. ALP-GFP was mislocalized to punc-

Figure 8. *kes1Δ* suppresses *drs2Δ* defects in TGN to vacuole protein trafficking. (A) *kes1Δ* suppresses a *drs2Δ* cs defect in the kinetics of CPY transport to the vacuole. WT, *drs2Δ*, *kes1Δ*, and *drs2Δ kes1Δ* cells were grown to mid-log phase in minimal medium at 30°C, half of the culture was shifted to 15°C for 15 min, and the cells were labeled with [³⁵S]methionine/cysteine for 10 min. Cells were chased with cold methionine/cysteine for the indicated times. CPY was recovered from each sample by immunoprecipitation and subjected to SDS-PAGE and autoradiography. (B) *kes1Δ* suppresses *drs2Δ dnf1Δ* defects in ALP localization. WT (BY4742), *drs2Δ dnf1Δ* (ZHY2149D), and *drs2Δ dnf1Δ kes1Δ* (BMY03) cells harboring pGO41 (GFP-ALP) were grown at 30°C to mid-log phase and examined by fluorescence microscopy. (C) Steady-state distribution of CPY and ALP precursor and mature forms. WT, *drs2Δ dnf1Δ*, *drs2Δ dnf1Δ kes1Δ* (same strains as in B), *gga1Δ gga2Δ* (KLY691), and *gga1Δ gga2Δ kes1Δ* (BMY40a) cells were grown in rich media at 30°C to mid-log phase. Whole cell lysates were prepared and immunoblotted to detect precursor (pro) and mature (m) forms of CPY and ALP.

tate structures outside the vacuolar membrane in *drs2Δ dnf1Δ* (Figure 8B, arrowheads). In contrast, ALP-GFP was properly targeted to the *drs2Δ dnf1Δ kes1Δ* vacuolar membrane (Figure 8B).

The steady-state levels of CPY and ALP precursor forms, which are normally transient and hard to detect by Western blot of WT cell lysates, were readily apparent in the *drs2Δ dnf1Δ* cells because of the trafficking defect. The accumulation of these precursors was completely suppressed in *drs2Δ dnf1Δ kes1Δ* cells (Figure 8C), indicating that *kes1Δ* suppressed the trafficking defect. The Gga1p and Gga2p clathrin adaptors are also implicated in the CPY and CPS transport pathway (Costaguta *et al.*, 2001). By comparison, *gga1Δ gga2Δ* showed a similar accumulation of p2 CPY as *drs2Δ dnf1Δ*, but this accumulation of the precursor was not suppressed in a *gga1Δ gga2Δ kes1Δ* strain. Thus, *kes1Δ* cells retain their dependence on GGAs for CPY transport but become less discriminate for the P4-ATPase requirement.

DISCUSSION

In this report, we provide mechanistic insight into the extreme cold-sensitive growth defect of *drs2Δ* cells and uncover a mutually antagonistic relationship between Drs2p and Kes1p. Genetic data indicate that Kes1p is hyperactive in *drs2Δ* and inhibits growth of these cells at low temperature, most likely through inhibition of Dnf P4-ATPases at the Golgi and their function in TGN-to-late endosome protein transport. Kes1p hyperactivity in *drs2Δ* cells also causes a substantial increase in the flux of exogenously applied sterol from the plasma membrane to sites of esterification and an alteration in the distribution of endogenous ergosterol. Kes1p also represses Drs2p function as deletion of *KES1* can suppress a *drs2-ts* allele and causes hyperactivity of Drs2p-dependent flippase activity in purified TGN membranes. Remarkably, flippase activity is attenuated *in vitro* by addition of picomolar concentrations of recombinant Kes1p to *kes1Δ* TGN membranes. We suggest that mutual repression between Kes1p and Drs2p provides a critical homeostatic mechanism for controlling the intracellular trafficking of both protein and sterol.

The recovery of *kes1Δ* in a screen for suppressors of *drs2Δ* cold-sensitive growth further emphasizes the importance of Drs2p to protein trafficking in the TGN/early endosome system and provides the first genetic link between *drs2Δ* and *sec14*. In addition to *kes1Δ*, other *sec14* suppressors include mutations in genes controlling the CDP-choline pathway for PC synthesis and *SAC1*, encoding a phosphoinositide phosphatase. Cold-sensitive growth of *drs2Δ* is not suppressed by a CDP-choline pathway mutation, but it is partially suppressed by *sac1Δ*. Therefore, the mechanism of *drs2Δ* suppression by *kes1Δ* is independent of PC biosynthesis. Stt4p, a plasma membrane PI 4-kinase, synthesizes most of the PI4P that accumulates in *sac1Δ* mutants. Accumulation of PI4P at non-TGN sites causes partial mislocalization of Kes1p from the TGN, thereby relieving its repressive effect on this organelle (Li *et al.*, 2002). This influence on Kes1p seems to account to the suppression of *sec14-ts* by *sac1Δ* and may also explain the suppression of *drs2Δ* by *sac1Δ*.

We had suggested previously that the inability of *drs2Δ* cells to grow at temperatures below 23°C was caused by a failure of the Dnf P4-ATPases to support an essential Drs2p function at the colder temperatures (Hua *et al.*, 2002). The current studies support this hypothesis, because suppression of *drs2Δ* growth defects by *kes1Δ* requires the presence of Dnf P4-ATPases. Importantly, *kes1Δ* cannot suppress *drs2Δ* defects in protein transport pathways that have a strict requirement for Drs2p. These include the formation of one exocytic vesicle class, and the AP-1/clathrin and Rcy1 pathways mediating transport between the TGN and early endosomes (Gall *et al.*, 2002; Liu *et al.*, 2008). In contrast, *drs2Δ* defects in ALP and CPY transport from the TGN to the vacuole, pathways supported by either Drs2p or Dnf P4-ATPases (Hua *et al.*, 2002), are suppressed by *kes1Δ*. The TGN is the likely point of suppression because Kes1p and Drs2p normally localize to the TGN, and this is presumably the last common site in the ALP and CPY transport pathways before these proteins arrive at the vacuole. Therefore, we suggest that removal of Kes1p improves Dnf function in the TGN so these P4-ATPases can better compensate for the loss of Drs2p.

The ability of *kes1Δ* to restore growth of *drs2Δ* cells at low temperature best correlates with the restoration of a wild-type rate of CPY and CPS transport from the Golgi to the vacuole at low temperature. The TGN → late endosome → vacuole route followed by these proteins is the only protein

trafficking pathway we have found that is normal in *drs2Δ* cells at permissive growth temperatures (Chen *et al.*, 1999) and defective at nonpermissive growth temperatures. This trafficking defect may be caused by a *cs* perturbation of a GGA/clathrin pathway. The GGA and AP-1 clathrin adaptors seem to mediate parallel pathways emanating from the TGN that deliver cargo to the late and early endosome, respectively (Black and Pelham, 2000; Costaguta *et al.*, 2001; Hirst *et al.*, 2001). Mutations in either pathway do not perturb growth of yeast; however, simultaneous loss of both pathways severely abrogates growth (Costaguta *et al.*, 2001; Hirst *et al.*, 2001). We had shown previously that *drs2Δ* cells exhibit a constitutive loss of the AP-1 pathway and that *drs2Δ gga1Δ gga2Δ* triple mutants are severely compromised for growth (Liu *et al.*, 2008). Here, we propose that the cold-sensitive defect in growth of *drs2Δ* cells is caused by a temperature-conditional defect in the GGA/clathrin pathway elicited only at low temperatures, combined with a constitutive defect in AP-1/clathrin function.

Why is Dnf function and the GGA/clathrin pathway perturbed at low temperature in *drs2Δ* cells, and how does loss of Kes1p suppress this defect? Important clues came from the analysis of ergosterol distribution and sterol trafficking in *drs2Δ* and *drs2Δ kes1Δ* cells. Relative to wild type, *drs2Δ* cells display reduced filipin staining of the plasma membrane and an altered sensitivity to nystatin, but no change in bulk ergosterol levels. These phenotypes are temperature independent and are suppressed by *kes1Δ*, suggesting that loss of Drs2p causes redistribution of ergosterol from the plasma membrane to internal membranes by a Kes1p-dependent mechanism. In fact, we found that *drs2Δ* cells exhibit a five- to sixfold increase in the rate of exogenously applied cholesterol esterification. This result implies an increased rate of transport from the plasma membrane to the ER, where the ACATs are localized. Elimination of Kes1p restored a wild-type rate of esterification in spite of the presence of six other Osh proteins in the *drs2Δ kes1Δ* cells. Thus, it seems that Kes1p is the only oxysterol binding protein homologue that is hyperactive in *drs2Δ* cells. These data support the proposed sterol transport function for Kes1p (Raychaudhuri *et al.*, 2006), although we cannot rule out the possibility that Kes1p regulates the activity of other proteins that directly mediate nonvesicular sterol transport. As a consequence of the altered sterol transport in *drs2Δ*, we suggest that either an increased concentration of sterol in the Golgi membrane, or an increased occupancy of Kes1p with sterol, inhibits the activity of P4-ATPases in the Golgi. In the case of *drs2Δ* cells, it is likely the combination of diminished membrane fluidity at low temperature and the repressive effect of Kes1p on Dnf P4-ATPases that disrupts protein transport in the CPY/GGA pathway. The observation that *drs2Δ* cells are high-pressure sensitive as well as cold-sensitive for growth suggests a lipid packing defect may contribute to this loss of membrane fluidity.

The molecular basis for hyperactivity of Kes1p in *drs2Δ* cells is not known. One possibility is that *drs2Δ* cells accumulate a sterol that is a potent activator of Kes1p. In support of this model, *drs2Δ* and *cdc50Δ* (nonglycosylated subunit) mutations exhibit a strong synthetic lethal relationship with *erg* mutations (Kishimoto *et al.*, 2005) that disrupt late steps in ergosterol synthesis (for example *erg6*) and accumulate ergosterol precursors. The synthetic lethality between *erg6Δ* and *drs2Δ* is completely suppressed by *kes1Δ*, indicating that Kes1p, but not other Osh proteins, is extremely toxic to *drs2Δ* cells producing sterols with an altered structure. *cdc50Δ* is synthetically lethal with *erg6Δ*, *erg2Δ*, *erg3Δ*, and *erg5Δ* (Kishimoto *et al.*, 2005), whose products catalyze in

series the conversion of zymosterol to ergosta-5-7-22-24(28)-tetraenol, the final intermediate to ergosterol. Accumulation of one or more of the intermediates in this pathway may stimulate the repressive activity of Kes1p at the Golgi. Consistently, cells depleted for Cdc50p and Erg3p massively accumulate Golgi and/or endosomal structures, reminiscent of strains deficient for Drs2 and Dnf P4-ATPases (Hua *et al.*, 2002; Kishimoto *et al.*, 2005).

However, it is also possible that Kes1p hyperactivity is a function of membrane disorganization rather than its sterol ligand. For example, Drs2p may help establish a plasma membrane structure with high-affinity for ergosterol, and therefore a low escape potential for this sterol, relative to the ER or Golgi (see Maxfield and Menon, 2006 and Lange and Steck, 2008 for discussion of cholesterol escape potential). Consistent with this possibility, loss of plasma membrane asymmetry in erythrocytes and fibroblasts has been shown to increase the escape potential of cholesterol from the plasma membrane (Lange *et al.*, 2007). In yeast, a membrane structure with low ergosterol escape potential may be established during budding of exocytic vesicles from the TGN as these vesicles are reported to have a concentration of ergosterol similar to the plasma membrane (300 μg ergosterol/mg protein; Daum *et al.*, 1998), much higher than what we find in TGN membranes (50–60 μg ergosterol/mg protein). This membrane organization may restrict the ability of Kes1p to bind and extract sterol from exocytic vesicles or the plasma membrane and redistribute it to internal membranes. In addition, ergosterol biosynthetic intermediates may have a reduced affinity for plasma membrane lipids, allowing a more efficient extraction from the plasma membrane (Li and Prinz, 2004; Lange and Steck, 2008). Thus, *drs2* Δ and *erg* perturbations may additively increase occupancy of Kes1p with sterol, thereby increasing its inhibitory effect on the remaining P4-ATPases. It does not seem to be the asymmetric distribution of PS and PE to the inner leaflet that is the critical structural feature of the plasma membrane needed to retain ergosterol. *dnf1,2,3* Δ cells exhibit a similar loss of PE and PS asymmetry as *drs2* Δ , and yet the plasma membrane of these cells stains normally with filipin. The *neo1-ts* mutant also stains normally with filipin (unpublished observations). Thus, Drs2p is the only P4-ATPase whose loss impacts sterol transport or localization, suggesting a unique and undefined influence of Drs2p on membrane organization and/or Kes1p activity.

The role of Drs2-related P4-ATPases in establishing a membrane structure that resists sterol extraction seems to be conserved through evolution and is medically relevant. The human disease progressive familial intrahepatic cholestasis (type I) is caused by mutations in the Atp8b1 P4-ATPase (also called FIC1) (Bull *et al.*, 1998), which has 41% amino acid sequence identity to Drs2p. A mouse model for Atp8b1 deficiency has been developed, and studies of these mice led to the proposal that loss of PS asymmetry at the bile canalicular membrane makes this membrane more sensitive to damage by the detergent effects of the excreted bile (Paulusma *et al.*, 2006; Cai *et al.*, 2009). Normally, cholesterol excretion into bile requires the action of the Abcg5/8 transporter, such that an *Abcg8*(–/–)-deficient mouse has a very low cholesterol output into bile. Surprisingly, mice deficient for both Atp8b1 and Abcg8 excrete wild-type levels of cholesterol into bile (Groen *et al.*, 2008). The *Abcg5/8*-independent excretion of bile in Atp8b1-deficient mice suggests that the disorganized structure of the bile canalicular membrane fails to retain cholesterol leading to its nonspecific extraction into the bile.

Elimination of Kes1p also suppresses the high-temperature growth defect caused by a *drs2-ts* allele in the absence of the Dnf P4-ATPases. This observation suggested that Kes1p represses the activity of Drs2p. In support of this possibility, we found a substantial increase in Drs2-dependent flippase activity in TGN membranes purified from *kes1* Δ cells. Addition of recombinant Kes1p to these membranes attenuated Drs2p activity back to wild-type levels. Remarkably, the half-maximal inhibitory effect of Kes1 was attained at a stoichiometry of ~ 1 –10 Kes1p molecules per 1000 Drs2p molecules. A 1000-fold increase in Kes1p concentration had no further inhibitory effect on flippase activity. Thus, the inhibitory influence of Kes1p on Drs2 activity is unlikely to be mediated by a direct protein-protein interaction between Kes1p and Drs2p. Consistently we have not been able to detect a direct interaction between Drs2p and Kes1p (unpublished observations). Inhibitory concentrations of Kes1p are also too low to significantly alter the ergosterol concentration of the TGN membrane preparation. It also seems unlikely that these low levels of Kes1p could effectively compete with other effectors for PI4P binding, which has been proposed to explain the negative influence of Kes1p on vesicle budding from the TGN (Li *et al.*, 2002).

The potency of the Kes1p attenuation of flippase activity suggests it is acting through an enzymatic intermediate, perhaps comparable with the signal transducing function of oxysterol binding protein (OSBP), a mammalian homologue of Kes1p. When bound to cholesterol, OSBP forms a complex with protein phosphatase 2A and a tyrosine phosphatase that attenuates signaling through the extracellular signal-regulated kinase (ERK) pathway. In response to 25-hydroxycholesterol binding, the OSBP phosphatase complex dissociates and OSBP translocates onto the Golgi (Wang *et al.*, 2005). Thus, it is possible that Kes1p attenuates Drs2p activity by regulating protein phosphorylation at the TGN. The Dnf P4-ATPases seem to be regulated by FPK1 and FPK2 protein kinases (Nakano *et al.*, 2008) although it is not known whether Drs2p is regulated by these or other protein kinases. Alternatively, Kes1p may regulate the activity of PI 4-kinases or 4-phosphatases that could secondarily influence flippase activity. Deletion of *KES1* has been shown to increase PI4P levels or availability at the TGN and suppresses growth defects caused by *pik1^{ts}* (Li *et al.*, 2002; Fairn *et al.*, 2007). The in vitro assay for Drs2p activity in purified TGN membranes should be amenable to testing these potential regulatory mechanisms of Kes1p.

Drs2p plays a critical role in vesicle budding from the TGN and early endosomes by a mechanism that is independent of coat recruitment. We proposed previously that the physical displacement of phospholipid from the luminal to cytosolic leaflet by Drs2p induces curvature in the membrane that is captured and molded by coat proteins into vesicles (Chen *et al.*, 1999; Graham, 2004; Liu *et al.*, 2008). It is logical that an appropriately balanced and coordinated activity between Drs2p and the vesicle budding machinery would be essential for the orderly segregation of protein and lipids into different transport pathways. Kes1p is well suited to regulate the activity of P4-ATPases in generating membrane curvature because it contains an ArfGAP lipid packing sensor (ALPS) domain that binds highly curved membranes (Drin *et al.*, 2007). The ALPS domain forms a lid over the sterol-binding pocket of Kes1p, suggesting that lipid packing density and/or membrane curvature may influence sterol binding to Kes1p.

We speculate that Drs2p imparts a degree of curvature to the TGN membrane that has an ideal set point for vesicle formation and for establishing sterol rich raft-like membrane

structures for export to the plasma membrane. As Drs2p flippase activity drives membrane curvature beyond this set point, Kes1p would sense the stress on the membrane and inhibit Drs2p activity until the membrane relaxes and inhibition is relieved. This model is consistent with the observed mode of Kes1p inhibition of Drs2p flippase activity: Kes1p potentially prevents hyperactivity of Drs2p but will not inhibit Drs2p activity below a basal level, even at very high concentrations of Kes1p. The membrane structure at this set point would also restrict the ability of sterol transfer proteins to extract ergosterol from the membrane. The set point model would explain why *drs2Δ* (reduced TGN flippase activity) and *kes1Δ* (enhanced TGN flippase activity) single mutants both display reduced filipin staining of the plasma membrane, whereas the double mutant seems more similar to wild-type cells. Although many aspects of this model remain to be tested, it provides a conceptual framework for understanding why a system of checks and balances between a P4-ATPase and an oxysterol binding protein may have evolved.

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REFERENCES

- Abe, F., and Minegishi, H. (2008). Global screening of genes essential for growth in high-pressure and cold environments: searching for basic adaptive strategies using a yeast deletion library. *Genetics* 178, 851–872.
- Alder-Baerens, N., Lisman, Q., Luong, L., Pomorski, T., and Holthuis, J. C. (2006). Loss of P4 ATPases Drs2p and Dnf3p disrupts aminophospholipid transport and asymmetry in yeast post-Golgi secretory vesicles. *Mol. Biol. Cell* 17, 1632–1642.
- Balasubramanian, K., and Schroit, A. J. (2003). Aminophospholipid asymmetry: a matter of life and death. *Annu. Rev. Physiol.* 65, 701–734.
- Bankaitis, V. A., Aitken, J. R., Cleves, A. E., and Dowhan, W. (1990). An essential role for a phospholipid transfer protein in yeast Golgi function. *Nature* 347, 561–562.
- Baumann, N. A., Sullivan, D. P., Ohvo-Rekila, H., Simonot, C., Pottekat, A., Klaassen, Z., Beh, C. T., and Menon, A. K. (2005). Transport of newly synthesized sterol to the sterol-enriched plasma membrane occurs via nonvesicular equilibration. *Biochemistry* 44, 5816–5826.
- Beh, C. T., Cool, L., Phillips, J., and Rine, J. (2001). Overlapping functions of the yeast oxysterol-binding protein homologues. *Genetics* 157, 1117–1140.
- Beh, C. T., and Rine, J. (2004). A role for yeast oxysterol-binding protein homologs in endocytosis and in the maintenance of intracellular sterol-lipid distribution. *J. Cell Sci.* 117, 2983–2996.
- Black, M. W., and Pelham, H. R. (2000). A selective transport route from Golgi to late endosomes that requires the yeast GGA proteins. *J. Cell Biol.* 151, 587–600.
- Bull, L. N., *et al.* (1998). A gene encoding a P-type ATPase mutated in two forms of hereditary cholestasis. *Nat. Genet.* 18, 219–224.
- Cai, S. Y., Gautam, S., Nguyen, T., Soroka, C. J., Rahner, C., and Boyer, J. L. (2009). ATP8B1 deficiency disrupts the bile canalicular membrane bilayer structure in hepatocytes, but FXR expression and activity are maintained. *Gastroenterology* 136, 1060–1069.
- Chen, C. Y., and Graham, T. R. (1998). An *arf1Δ* synthetic lethal screen identifies a new clathrin heavy chain conditional allele that perturbs vacuolar protein transport in *Saccharomyces cerevisiae*. *Genetics* 150, 577–589.
- Chen, C. Y., Ingram, M. F., Rosal, P. H., and Graham, T. R. (1999). Role for Drs2p, a P-type ATPase and potential aminophospholipid translocase, in yeast late Golgi function. *J. Cell Biol.* 147, 1223–1236.
- Chen, S., Wang, J., Muthusamy, B. P., Liu, K., Zare, S., Andersen, R. J., and Graham, T. R. (2006). Roles for the Drs2p-Cdc50p complex in protein transport and phosphatidylserine asymmetry of the yeast plasma membrane. *Traffic* 7, 1503–1517.
- Chen, S. H., Chen, S., Tokarev, A. A., Liu, F., Jedd, G., and Segev, N. (2005). Ypt31/32 GTPases and their novel F-box effector protein Rcy1 regulate protein recycling. *Mol. Biol. Cell* 16, 178–192.
- Costaguta, G., Stefan, C. J., Bensen, E. S., Emr, S. D., and Payne, G. S. (2001). Yeast Gga coat proteins function with clathrin in Golgi to endosome transport. *Mol. Biol. Cell* 12, 1885–1896.
- Daum, G., Lees, N. D., Bard, M., and Dickson, R. (1998). Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*. *Yeast* 14, 1471–1510.
- Drin, G., Casella, J. F., Gautier, R., Boehmer, T., Schwartz, T. U., and Antonny, B. (2007). A general amphipathic alpha-helical motif for sensing membrane curvature. *Nat. Struct. Mol. Biol.* 14, 138–146.
- Elvington, S. M., Bu, F., and Nichols, J. W. (2005). Fluorescent, acyl chain-labeled phosphatidylcholine analogs reveal novel transport pathways across the plasma membrane of yeast. *J. Biol. Chem.* 280, 40957–40964.
- Fairn, G. D., Curwin, A. J., Stefan, C. J., and McMaster, C. R. (2007). The oxysterol binding protein Kes1p regulates Golgi apparatus phosphatidylinositol-4-phosphate function. *Proc. Natl. Acad. Sci. USA* 104, 15352–15357.
- Fairn, G. D., and McMaster, C. R. (2008). Emerging roles of the oxysterol-binding protein family in metabolism, transport, and signaling. *Cell Mol. Life Sci.* 65, 228–236.
- Fang, M., Kearns, B. G., Gedvilaite, A., Kagiwada, S., Kearns, M., Fung, M. K., and Bankaitis, V. A. (1996). Kes1p shares homology with human oxysterol binding protein and participates in a novel regulatory pathway for yeast Golgi-derived transport vesicle biogenesis. *EMBO J.* 15, 6447–6459.
- Fei, W., Alfaro, G., Muthusamy, B. P., Klaassen, Z., Graham, T. R., Yang, H., and Beh, C. T. (2008). Genome-wide analysis of sterol-lipid storage and trafficking in *Saccharomyces cerevisiae*. *Eukaryot. Cell* 7, 401–414.
- Furuta, N., Fujimura-Kamada, K., Saito, K., Yamamoto, T., and Tanaka, K. (2007). Endocytic recycling in yeast is regulated by putative phospholipid translocases and the Ypt31p/32p-Rcy1p pathway. *Mol. Biol. Cell* 18, 295–312.
- Gall, W. E., Geething, N. C., Hua, Z., Ingram, M. F., Liu, K., Chen, S. I., and Graham, T. R. (2002). Drs2p-dependent formation of exocytic clathrin-coated vesicles in vivo. *Curr. Biol.* 12, 1623–1627.
- Gall, W. E., Higginbotham, M. A., Chen, C., Ingram, M. F., Cyr, D. M., and Graham, T. R. (2000). The auxilin-like phosphoprotein Swa2p is required for clathrin function in yeast. *Curr. Biol.* 10, 1349–1358.
- Gietz, R. D., and Woods, R. A. (2006). Yeast transformation by the LiAc/SS Carrier DNA/PEG method. *Methods Mol. Biol.* 313, 107–120.
- Goodson, H. V., Anderson, B. L., Warrick, H. M., Pon, L. A., and Spudich, J. A. (1996). Synthetic lethality screen identifies a novel yeast myosin I gene (MYO5): myosin I proteins are required for polarization of the actin cytoskeleton. *J. Cell Biol.* 133, 1277–1291.
- Graham, T. R. (2001). Metabolic labeling and immunoprecipitation of yeast proteins. *Curr. Protoc. Cell Biol.* Chapter 7, Unit 7.6.
- Graham, T. R. (2004). Flippases and vesicle-mediated protein transport. *Trends Cell Biol.* 14, 670–677.
- Groen, A., *et al.* (2008). Abcg5/8 independent biliary cholesterol excretion in Atp8b1-deficient mice. *Gastroenterology* 134, 2091–2100.
- Hanover, J. A., Forsythe, M. E., Hennessey, P. T., Brodigan, T. M., Love, D. C., Ashwell, G., and Krause, M. (2005). A *Caenorhabditis elegans* model of insulin resistance: altered macronutrient storage and dauer formation in an OGT-1 knockout. *Proc. Natl. Acad. Sci. USA* 102, 11266–11271.
- Hirst, J., Lindsay, M. R., and Robinson, M. S. (2001). GGAs: roles of the different domains and comparison with AP-1 and clathrin. *Mol. Biol. Cell* 12, 3573–3588.
- Hua, Z., Fatheddin, P., and Graham, T. R. (2002). An essential subfamily of Drs2p-related P-type ATPases is required for protein trafficking between Golgi complex and endosomal/vacuolar system. *Mol. Biol. Cell* 13, 3162–3177.
- Im, Y. J., Raychaudhuri, S., Prinz, W. A., and Hurley, J. H. (2005). Structural mechanism for sterol sensing and transport by OSBP-related proteins. *Nature* 437, 154–158.

- Jiang, B., Brown, J. L., Sheraton, J., Fortin, N., and Bussey, H. (1994). A new family of yeast genes implicated in ergosterol synthesis is related to the human oxysterol binding protein. *Yeast* 10, 341–353.
- Kaplan, M. R., and Simoni, R. D. (1985). Transport of cholesterol from the endoplasmic reticulum to the plasma membrane. *J. Cell Biol.* 101, 446–453.
- Kishimoto, T., Yamamoto, T., and Tanaka, K. (2005). Defects in structural integrity of ergosterol and the Cdc50p-Drs2p putative phospholipid translocase cause accumulation of endocytic membranes, onto which actin patches are assembled in yeast. *Mol. Biol. Cell* 16, 5592–5609.
- Lange, Y., and Steck, T. (2008). Cholesterol homeostasis and the escape tendency (activity) of plasma membrane cholesterol. *Prog. Lipid Res.* 47, 319–332.
- Lange, Y., Ye, J., and Steck, T. L. (2007). Scrambling of phospholipids activates red cell membrane cholesterol. *Biochemistry* 46, 2233–2238.
- Li, X., Rivas, M. P., Fang, M., Marchena, J., Mehrotra, B., Chaudhary, A., Feng, L., Prestwich, G. D., and Bankaitis, V. A. (2002). Analysis of oxysterol binding protein homologue Kes1p function in regulation of Sec14p-dependent protein transport from the yeast Golgi complex. *J. Cell Biol.* 157, 63–77.
- Li, Y., and Prinz, W. A. (2004). ATP-binding cassette (ABC) transporters mediate nonvesicular, raft-modulated sterol movement from the plasma membrane to the endoplasmic reticulum. *J. Biol. Chem.* 279, 45226–45234.
- Liscum, L., and Munn, N. J. (1999). Intracellular cholesterol transport. *Biochim. Biophys. Acta* 1438, 19–37.
- Liu, K., Hua, Z., Nepute, J. A., and Graham, T. R. (2007). Yeast P4-ATPases Drs2p and Dnf1p are essential cargos of the NPFXD/Slal1p endocytic pathway. *Mol. Biol. Cell* 18, 487–500.
- Liu, K., Surendhran, K., Nothwehr, S. F., and Graham, T. R. (2008). P4-ATPase requirement for AP-1/clathrin function in protein transport from the trans-Golgi network and early endosomes. *Mol. Biol. Cell*
- Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14, 953–961.
- Maxfield, F. R., and Menon, A. K. (2006). Intracellular sterol transport and distribution. *Curr. Opin. Cell Biol.* 18, 379–385.
- McGee, T. P., Skinner, H. B., Whitters, E. A., Henry, S. A., and Bankaitis, V. A. (1994). A phosphatidylinositol transfer protein controls the phosphatidylcholine content of yeast Golgi membranes. *J. Cell Biol.* 124, 273–287.
- Morozova, N., et al. (2006). TRAPP2 subunits are required for the specificity switch of a Ypt-Rab GEF. *Nat. Cell Biol.* 8, 1263–1269.
- Mousley, C. J., Tyeryar, K. R., Vincent-Pope, P., and Bankaitis, V. A. (2007). The Sec14-superfamily and the regulatory interface between phospholipid metabolism and membrane trafficking. *Biochim. Biophys. Acta* 1771, 727–736.
- Nakano, K., Yamamoto, T., Kishimoto, T., Noji, T., and Tanaka, K. (2008). Protein kinases Fpk1p and Fpk2p are novel regulators of phospholipid asymmetry. *Mol. Biol. Cell* 19, 1783–1797.
- Natarajan, P., and Graham, T. R. (2006). Measuring translocation of fluorescent lipid derivatives across yeast Golgi membranes. *Methods* 39, 163–168.
- Natarajan, P., Wang, J., Hua, Z., and Graham, T. R. (2004). Drs2p-coupled aminophospholipid translocase activity in yeast Golgi membranes and relationship to in vivo function. *Proc. Natl. Acad. Sci. USA* 101, 10614–10619.
- Novick, P., Field, C., and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* 21, 205–215.
- Paulusma, C. C., et al. (2006). Atp8b1 deficiency in mice reduces resistance of the canalicular membrane to hydrophobic bile salts and impairs bile salt transport. *Hepatology* 44, 195–204.
- Pomorski, T., Lombardi, R., Riezman, H., Devaux, P. F., van Meer, G., and Holthuis, J. C. (2003). Drs2p-related P-type ATPases Dnf1p and Dnf2p are required for phospholipid translocation across the yeast plasma membrane and serve a role in endocytosis. *Mol. Biol. Cell* 14, 1240–1254.
- Raychaudhuri, S., Im, Y. J., Hurley, J. H., and Prinz, W. A. (2006). Nonvesicular sterol movement from plasma membrane to ER requires oxysterol-binding protein-related proteins and phosphoinositides. *J. Cell Biol.* 173, 107–119.
- Rivas, M. P., Kearns, B. G., Xie, Z., Guo, S., Sekar, M. C., Hosaka, K., Kagiwada, S., York, J. D., and Bankaitis, V. A. (1999). Pleiotropic alterations in lipid metabolism in yeast *sac1* mutants: relationship to “bypass Sec14p” and inositol auxotrophy. *Mol. Biol. Cell* 10, 2235–2250.
- Saito, K., Fujimura-Kamada, K., Furuta, N., Kato, U., Umeda, M., and Tanaka, K. (2004). Cdc50p, a protein required for polarized growth, associates with the Drs2p P-type ATPase implicated in phospholipid translocation in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 15, 3418–3432.
- Schaaf, G., et al. (2008). Functional anatomy of phospholipid binding and regulation of phosphoinositide homeostasis by proteins of the sec14 superfamily. *Mol. Cell* 29, 191–206.
- Sherman, F. (1991). Getting started with yeast. *Methods Enzymol* 194, 3–21.
- Stevens, T., Esmon, B., and Schekman, R. (1982). Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell* 30, 439–448.
- van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008). Membrane lipids: where they are and how they behave. *Nat. Rev. Mol. Cell Biol.* 9, 112–124.
- Wang, P. Y., Weng, J., and Anderson, R. G. (2005). OSBP is a cholesterol-regulated scaffolding protein in control of ERK 1/2 activation. *Science* 307, 1472–1476.